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THREE FEEDING STIMULANTS IN THE ALFALFA STEAM DISTILLATE FOR THE ALFALFA WEEVILS, (HYPERA POSTICA (GYL.))

by

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INTRODUCTION

The alfalfa weevil, Hypera postica, was first discovered in the United States in the Salt Lake City Utah area in 1904[1,2], and in the East in Maryland in 1951[3]. The western strain spread to the east slowly, reaching Kansas by the end of 1960. The Eastern strain spread rapidly westward, reaching Kansas by 1970. In 1974 at least 95% of the alfalfa acreage in Kansas was infested. It is now found in all parts of this country infesting alfalfa in nearly every state and is one of the most important pests of alfalfa[4]. Though, chemicals are being used to control this insect[5], they have the following disadvantage: (1) development of resistance (2) the persistance of residues of certain insecticides may contribute to foliation of the environment (3) the high costs involved in production and application and (4) they may be harmful not only to the target pest, but also to beneficial insects, wildlife and to humans as well[6].

One alternative of biological control can be chosen by investigating the chemicals responsible for attracting, stimulating or inhibiting the weevil during feeding and/or ovipositing, and then using that information to develop alfalfa cultivates resistant to the attack of alfalfa weevils. The purposes of this research were to look for (1) the feeding and/or ovipositing stimulants which might be present in alfalfa (2) the ovipositing deterrent which might be present in red clover.

THE ALFALFA WEEVIL, family Curculionidae, moves by flying, crawing and hitch-hiking (by riding along with the hay during harvest). It usually produces only one generation during the growing season unless there is an especially long and warm fall when a second partial generation is produced. In a Southern States a second generation may occur due to their warmer climate. The alfalfa

weevil undergoes a holometabolic life cycle with eggs, larvae, instans pupa and adult.

The eggs are quite small, being about 1/32 of an inch long. They are oval in shape and are light yellow in color when first deposited later turning a darker yellow. The eggs are laid in fresh alfalfa stems in clusters varying from 1 to 58 with an average of 14 eggs per cluster. They are laid either in the spring and will hatch in one to two weeks, or in the fall when they will overwinter. The length of time needed for hatching depends on the temperature, with the optimum in the range of $75^{o}F$ to $85^{o}F$.

The larvae of the alfalfa weevil, just after hatching, are slightly longer than 1/32 inch, legless, yellowish-tan in color with a black head. The larvae then either emerge from the puncture and climb outside of the stem to the buds or migrate over the soil surface to its host plant. The larvae feed on the foliage of alfalfa beginning with the youngest terminal leaves and working down the stem. The damaged fields take on a grayish to whitish cast that is somewhat similar to severe frost damage.

After the larvae have fed for three to four weeks, they become greener and about 3/8 inch long. After the third molting, the larvae are green with a white line along the middle of the back and have a dark brown head. Since the larvae are not highly mobile, a successful choice of larvae host plant has to be made by the ovipositing females.

Before pupation the full grown larvae spin a white, threadlike cocoon. The pupa is light green when it is first formed and it lasts one to two weeks[7,8]. As it becomes darker, legs, back and wing covers of the adult can be seen. The pupa is found on the lower portion of the plant, in dead leaves, ground litter

and on the bare earth.

The adult is light brown with a dark line starting from the head running down to the back. They grow darker as they age, have a short distinct snout, and are about 3/16 inch long at maturity. The adults may feed on both stems and leaves of alfalfa and an average life span is ten to fourteen months.

Feeding by the adult is not as extensive as in the larval stage. The weevils enter diapause during June, July and August. After this is broken, they sexually mature, start mating and feeding in the field.

According to their host range the phytophagous, or herbivorous insects are classified into polyphagous, oligophagous and monophagous insects.

To the polyphagous insects, many different plant species from different families can be fed on. To the oligophagous, only some plant species belonging to a few related families are acceptable. The monophagous, only selects one plant species, such as alfalfa weevils to alfalfa plants.

HOST SELECTION, food finding and food acceptance, by insects seems to be governed by plant-produced stimulants. In general, a plant is chosen because it represents the msot suitable medium for nutrition and propagation of the pest insect [9-12].

Host plant selection may be partitioned into 3 phases: recognition, feeding response and oviposition.

(1) "RECOGNITION": It is still questionable whether the progeny has a preference for that particular host after the parents as adults or larvae had been confined to it for one or a few generations.

(2) "FEEDING RESPONSE"[13-17]: The acuity of odor-perception of insects far exceeds that of man, and they rely greatly on olfactory perception in searching

for food and mating[18,19]. Thus, the odor of a given plant may act as an attractant or as an arrestant.

Having encountered a plant, an insect then must determine whether this plant provides a satisfactory site for feeding and/or ovipositing. An insect, moving over the plant surface, may be "arrested" by an odor or chemical perceived by contact chemoreceptors.

If hungry enough, it may take a test bite and the odor perceived from the injured plant and the chemicals ingested determine whether the feeding is to continue. It is more likely that the feeding refuse is based on a series of stimuli rather than on only one single attractant that entirely determines the choice. However, the absence or presence of one critical factor may affect the choice.

(3) "OVIPOSITION": The oviposition response and the factors affecting it are of vital importance to the insects. The stimuli affecting oviposition in an insect are both internal (state of nutrition, age of the insect, fertility of the insect and the endogenous rhythms) and external (temperature, humidity, light, air and availability of water)[20].

Some insects select plant-hosts especially for the purpose of oviposition, and in some cases it is the adult female seeking an oviposition site[21,22]. The orientation of an insect to a host for the purpose of laying eggs has been shown to be directed in some cases, by olfactory mediation[23-26].

The oviposition behavior of the insect can also be affected by repellent substances. In some instances these repellent substances are deposited at the site of egg-laying by an ovipositing female to prevent other females of the same species from laying eggs in the same place[27-29].

Chemosensory receptors are classified as contact or olfactory receptors and are found on the thorax, antennae, legs, mouth parts or scattered over the general body surface of the insect.

These receptors, which must receive plant stimuli, are known to affect the orientation, feeding and reproductive behaviors of the insects and not the same for all animals[30].

Olfaction. General insect species are able to recognize the odor of their food plant by a sense of smell over distances as far as forty centimeters[31]. The affect of plant odor seems critical in the first step of the feeding process.

The removal of part of all of this appendages in the antennae can cause an insect to make a test bite on the normally rejected food.

Gustation. Dithier[32] and Hodgson[33] found that the taste hairs of several species of blow flies, butterflies, and honey bees[34], generally contain a sugar-sensitive cell, a salt sensitive cell and mechanoreceptor. For blow flies, even a water-sensitive cell and a protein-sensory cell are present[35]. More detailed analysis reveals evidence that in the process of food selection both phagostimulant and deterrents are involved.

LITERATURE REVIEW: Byrne[36] tested 10 Medicago and related species on adult feeding, oviposition and larval sfurvival. He found that a poor host for the larva also was a poor host for the adult and that the closer the plant was related to Medicago or Melilotus (sweetclover), the more suitable a host it was. He also found that alfalfa, sweetclover and red clover were acceptable to the alfalfa weevil adults, however only the first two could be fed to the larvae and only the alfalfa plant was attacked at an economic destructive level.

He also found that the ovipositing females would not feed or oviposit on

hop clover or hebit in the absence of alfalfa while they did lay a great number of eggs in these two non-host if they were offered together with alfalfa plants. Henbit and hop clover provided suitable tactile or visual stimuli for oviposition but it was important to have alfalfa present. The larvae were found not to feed on henbit nor on red clover.

In 1969, Byrne[36] ground fresh alfalfa plants and filtered the juice. He then dipped red clover into this juice, the filtrate, and fed it to the alfalfa weevil adult. The red clover stem was dissected and the number of eggs was recorded. He found that the alfalfa juice did increase the number of eggs laid be the alfalfa weevils. (Table 1) The ratio between eggs deposited in red clover and the red clover dipped in alfalfa juice was 1 to 3.35. That suggested that alfalfa juice contains an ovipositing stimulant.

He then exposed the stems of alfalfa plants covered with red clover stems to the alfalfa weevil adults and the number of eggs laid inside the stem was recorded. He found this number (Table 2) was greatly decreased by covering the stems of the alfalfa plants with the red clover stems. The ratio between eggs deposited in alfalfa stems and those in alfalfa stems covered with red clover stems suggested that oviposition inhibitors in red clover prevailed over the

TABLE 1

Treatment	# of eggs laid
Alfalfa	5094
Red clover	233
Red clover dipped in alfalfa juice	876

TABLE 2

Treatment	# of eggs laid
Alfalfa	266
Alfalfa with stems covered with red clover stems	32
Red clover	3

ovipositing stimulants in alfalfa. He tried to feed fresh alfalfa plants extracted with steam distillation or ether to alfalfa weevil adults. Only the steam distillate of fresh alfalfa showed a positive though not very exciting response. He then diluted the steam distillate with water to 0.1%, 0.05%, 0.025% and 0.0125% by volume. These dilutions as well as the concentrated fresh alfalfa extract, the fresh alfalfa steam distillate, and distilled water as the control, were tested by applying four drops of the test solution on to a section of pith, which was placed in the center of a petri dish covered with a nylon mesh. In a first series of experiments, the weevils were prestarved without access to water. In this condition orientaion of the weevils towards water was marked. Thereafter in a second experiment, the weevils were offered water during the prestarvation period. The results of both tests are summarized in Table 3.

Weevils which had water available during the pre-test starvation period showed a marked decrease in orientaion to the pith segments. The highest average number of weevils oriented to the pith was to a concentration of 0.025% of the alfalfa steam distillate, regardless of the presence of water during the starvation period. He also found that the attractant must be dissovlved in water to elicit a response.

TABLE 3

	Conc. of alfalfa extract %	Average number of weevils oriented to test material*	Conclusion
Water not offered previously	100	0.00	repellent
	0.1	2.50	attractive
	0.05	3.4	very attractive
	0.025	3.65	most attractive
	0.0125	1.40	indifferent
	distilled water	2.05	attractive
Water offered previously	0.1	0.43	indifferent
	0.05	0.53	indifferent
	0.025	2.67	very attractive
	distilled water	0.40	not attractive

* Average of all counts in all combinations; 8-10 replications/combination; 15 counts/replication.

RESPONSES OF ALFALFA WEEVILS IN OPEN CAGE TESTS TO VARIOUS CONCENTRATIONS OF ALFALFA EXTRACT. Weevils on test dishs were counted at 1 minute intervals; 10 weevils per replication.

In another study, Byrne[36] extracted fresh alfalfa with various solvents, hexane, diethyl ether, 70% ethanol at 27°C and at 60°C and water at 27°C and at 70°C, plus a steam distillate. He then dipped red clover into these alfalfa extracts and observed oviposition. Since none of them were bioactive, he concluded that none of these alfalfa extracts contained an oviposition stimulant.

He then tried several different solvent extractions by using diethyl ether, hexane and water and observed (Table 4) oviposition by the alfalfa weevils. These results showed that the hexane extract reduced the oviposition rate by 75% of that found in the alfalfa dipped in hexane control. While this value for hexane itself was only 25%. Also, because the other extractions do not lower the oviposition level, it appeared that the bioactive material in the red clover did not dissolve in either diethyl ether or in water, but in hexane. He concluded that it was relatively non-polar[37].

TABLE 4

Treatment	# of eggs laid
Alfalfa dipped in red clover hexane extract	297
Hexane control	850
Alfalfa dipped in red clover ether extract	1090
Alfalfa dipped in red clover ether control	960
Alfalfa only	1150
Red clover	10
Alfalfa dipped in water control	1150
Alfalfa dipped in water extract of red clover	960

Then, Byrne[36] separated the red clover juice into two fractions supernatant and sediment by centrifugation at 1500 g. He fed the alfalfa weevils with (1) alfalfa (2) alfalfa plus supernatant (3) alfalfa plus sediment and (4) red clover. The number of eggs laid inside the stems (Table 5) showed that the sediment produced by centrifugation at 1500 g for 15 minutes does

contain most of that fraction of the juice which prevents attainment of normal levels of oviposition by the alfalfa weevils in alfalfa.

TABLE 5

Treatment	# of eggs laid
Alfalfa	4182
Alfalfa + supernatant of red clover	3014
Alfalfa + sediment of red clover	1516
Red clover	210

From Table 5, there is evidence that an oviposition deterrent is present in the red clover. This research involved examing the steam distillate for feeding and oviposition stimulants and a preliminary study was made to isolate any ovipositing deterrent in the red clover.

EXPERIMENTAL

In this research, a steam distillate of alfalfa and a hexane extract of red clover were prepared and fed to the alfalfa weevils for feeding and/or ovipositing tests. Three compounds were then identified and examined as feeding and/or ovipositing stimulants.

I. Material and Reagents

The hexane used in this study was purified by Vogel's[38] procedures. All of the other reagents used were analytical grade and commercial compounds were used without further purification.

The alfalfa plants, Kansas Common, were collected from a field at College Avenue in Manhattan, Kansas. Over winter alfalfa from the same field was transplanted in a greenhouse which had an eight hour light period. Red clover plants were either collected from a field along highway 24 or one in front of the St. Mary Hospital in Manhattan, Kansas. During the winter, the red clover plants were grown from seed in the greenhouse at Kansas State University. Red clover plants were selected for the same stem thickness. The stems were picked randomly over the field.

The alfalfa weevils were obtained from a colony either from Illinois or Kansas and maintained in the Entomology Department of the Kansas State University. Weevils dieing during the experiment were replaced by adults of the same sex.

The TLC plates coated with silica gel and fluorecent indicator were purchased from Fisher Co. (Catalogue No. 6-601 C).

All the tests were conducted in a growth chamber with a light period of eight hours. The temperature was maintained at 72°F during the "day" and 68°F

at night. The positions of the storage boxes in the growth were always chosen randomly.

II. Procedures

1. The Alfalfa Steam Distillate

(A) Preparation of the alfalfa steam distillate

One hundred grams of fresh alfalfa leaves, together with 100 ml of distilled water were blended at medium speed in a blender for one minute, squeezed through a piece of cheese cloth and the filtrate placed in a 250 ml round bottom flask and steam distilled. The rate of distillation was adjusted and the distillate collected in an icebathed flask.

It was suggested[39] to harvest the alfalfa plants after 10 am to get a maximum content of the volatiles, since the contents of the volatile compounds are variable during the day.

As the concentrations of the volatiles present in the alfalfa steam distillate decreased by slow evaporation, the steam distillate was always kept in a refrigerator no longer than 10 days.

(B) Identification of the compounds in the steam distillate of alfalfa

The technique used for the identification of these volatile compounds was GC-MS spectroscopy. Porapak packings can handle water very well. A 1/8 inch diameter, 6 foot long stainless steel column, coiled to fit the GC oven, was packed with a mixture of Porapaks Q and R at a ratio of 4 to 1 by weight. This ratio was found to be very effective for the separation of the alfalfa steam distillate.

The GC-MS work was done at Midwest Reaseach Institute in Kansas City, Missouri. The Mass Spectrometer used was a Varian Mat-CH 4 Model. The column was the same one used for the separation study.

To avoid too much water entering the Mass Spectrometer after the injection of the sample, the system was vented for five minutes then the sample was introduced into the Mass Spectrometer. Printouts of the compounds and a listing of the peaks and their relative intensities were obtained.

The three unidentified compounds of the first six peaks in the GC chromatogram, were identified. (Figure 3, Karen Curry[41] identified peaks #3, 4 and 6.) They were then confirmed with GC retention times. The gas chromatograph used was a Bendix Model 2200 with both flame ionization and thermal conductivity detectors. The conditions of the GC work are shown in Table 6 and a typical GC chromatogram is shown in Figure 3.

TABLE 6

Sample size	3.5 1 to 5 1.
Carrier gas	helium, 22.6 ml/minute.
Column length	6 foot in length, 1/8 inch in diameter.
Packing material	Porapaks Q and R at a ratio of 4 to 1.
Column condition	temperature programmed.
Initial temperature	60°C.
Final temperature	200°C.
Program rate	10°C/minute.
Injection port temperature	247°C.
Detector temperature	250°C.
Detector	flame ionization detector.
Sensitivity	20 or 50 .

The steam distillate of the alfalfa was then spiked with a small amount of the identified compounds one at a time. The retention times of the identified compounds were matched with the identified peaks. The retention times were double checked by using a Porapak QS column under the same conditions of the Porapak Q and R mixture column.

(C) Standard solutions of the identified compounds

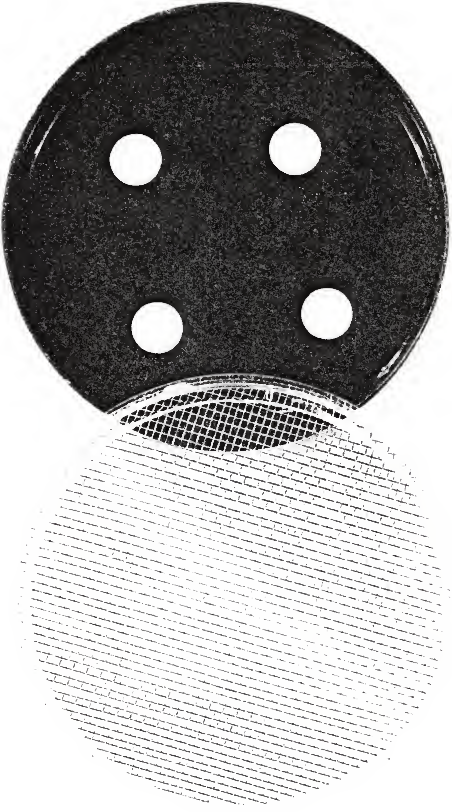
Five microliters of the alfalfa steam distillate were injected into the gas chromatograph, the peak area of each component was calculated and the concentration of each identified component roughly estimated. Then, several dilutions of a known concentration were prepared. Five microliters of these prepared dilutions were injected and the respective peak areas were calculated, A calibration curve was obtained by plotting the concentration against peak area. By interpolation, the concentration of the identified compounds could be found. These concentrations were then taken as standard concentrations (100%), and several dilutions 0.1%, 0.05%, 0.025% and 0.0125% were made.

(D) Olfactory orientation test....... feeding tests

In this study, unsexed alfalfa weevils were used. Only water was provided for 24 hours before the test started. One drop of either one of the test solutions or water was applied to a filter paper (Fig. 1). Five weevils were used in each of the six replicates.

The petri dishes were painted black and completely dried, to avoid interferences from the light, from objects surrounded the dishes, or from the volatiles from the petri dish. Four pieces of filter paper (1.0 cm in diameter away from the edge). Two of these four pieces of filter paper were reserved

Setup for feeding tests



Figures 1

for the test solution and another two for water were randomly selected. The lids of the petri dishes were punched in the center (Figure 1) and the hole was covered with a copper screen, to help decrease the accumulation of the volatiles. The lowest concentration was examined first, then proceeding to the higher concentrations.

The standard solutions of all six compounds together with the dilutions of 0.1%, 0.05%, 0.025% and 0.0125%, were bilassayed for olfactory orientation.

After dropping the solution or water on the filter paper, the weevils were placed in the center of the petri dish and the number of weevils reaching the filter paper recorded in 30 second intervals. The test periods lasted eight minutes. All six replicates for the same test solution were done before going to another higher concentration. Two replicates were conducted at the same time.

(E) Ovipositing tests

In this study, only those concentrations showing the highest feeding response in section D for each solution and the 100% mixture were tested. The setup is shown in Figure 2. A wheat germ diet[40] (Table 7,) was formulated into cubes (1 cm^3) and provided the only food source. Without this diet the weevils would just stop laying eggs because of the insufficiency of necessary nutrition.

All of the alfalfa weevils used in this experiment were sexed and for 24 hours before the test were fed only with diet cubes. Five of each sex were transfered into a cardboard box containing the prepared alfalfa stems and covered with a petri dish top.

Freshly prepared solutions and alfalfa stems were provided every other

TABLE 7

Compotition of the artificial diet for rearing the alfalfa weevils, <u>Hypera Postica</u>[40].

Constituent*	gram per 100 ml diet
Lysine (vitamin free)	3.00
Lactoalbumin hydrolysate	1.00
Sucrose	5.00
Yeast extract	1.00
Salt mixture, Wesson's	1.00
Choline chloride	0.10
Cholesterol	0.15
Corn oil	0.50
Ascorbic acid	0.40
Potassium hydroxide	0.30
Agar	3.00
Wheat germ powder	10.00
Alfalfa leave powder	10.00
Sorbic acid	0.15
Methyl-p-hydroxybenzoate	0.10
Sarptomycine sulfate	0.05
Benlate	0.05
Water to 100 ml	

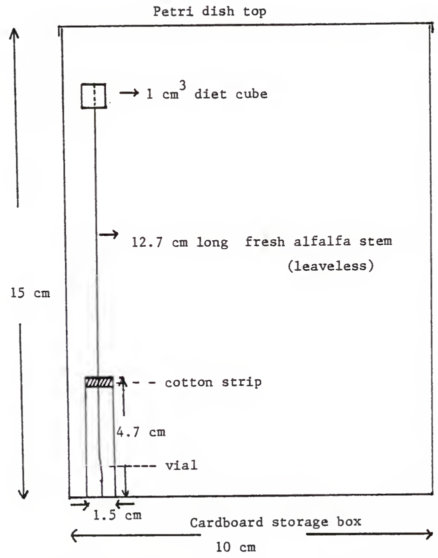
* All constituents were from Bioserve Co., New Jercy.

day, while the diet cubes were changed daily to prevent dryness. The stems were then dissected and the number of eggs laid were counted. The prepared diet cubes were prepared weekly and kept in the refrigerator. The test period was three weeks.

A fresh alfalfa stem (about 0.2 cm in diameter and 12.7 cm in length), with one diet cube on the top (Figure 2), was inserted into a vial which was covered with a cotton strip on top. Two different setups were used in this experiment. (1) The vials were filled with either one of the test solutions for the test groups or water for the control group. (2) The vials were filled with water for both the test groups and the control. However, while preparing the diet cubes, these test solutions took the place of water for the respective test groups. That is, the diet cubes used in this setup were different for each test groups and the control group.

Figure 2

Setup for ovipositing tests



In araangement 1: test solution or water contained in the vial and the diet cubes were prepared by following the formula.

In arrangement 2: water contained in all vials. The test solutions were mixed with diet to replace the necessary water.

2. The Hexane Extract of Red Clover

Byrne[36] reported that the deterrent in the red clover could be extracted by hexane, but not diethyl ether nor water. The first step of this research was to show the effectiveness of the hexane extract of red clover, then determine what compound or compounds act as deterrents.

(A) Preparation of hexane extract of red clover

Three hundred grams of fresh red clover, together with 300 ml of distilled water, were blended at mijium speed for 1 minute and then filtered through a piece of 100% cheese cloth. The filtrate was separated into two fractions, sediment and supernatant, by centrifugation at 1500 g for 30 minutes at $5^{o}C$.

The supernatant was discarded and the sediment was washed alternately with diethyl ether and water in a Soxhlet extractor for 36 hours, then suction dried for two hours with the help of a water aspirator. The sediment, about 5 grams, was then further extracted for 36 hours in a Soxhlet extractor with hexane. The hexane extract was concentrated to a volume of 25 ml by a rotary evatorator. The effectiveness of this hexane extract was then determined by the previously described bioassay for oviposition.

(B) Oviposition test for the hexane extract of red clover

(1) Study of the history of alfalfa weevils

The ovipositing bioassay is a very difficult experiment, because the behavior of each individual alfalfa weevil is so different. It was very important to find out what their behavious right before the tests.

Eight-four alfalfa weevils of each sex were divided into six groups and fed with fresh alfalfa. Two, six inch long alfalfa stems with leaves were

wrapped with one 1 cm wide cotton strip at a one inch height from the bottom, and were inserted into a vial which was filled with distilled water.

The alfalfa stems were changed every Monday, Wednesday and Friday. After the test period of two weeks, the stems were dissected and the number of eggs in each stem recorded. The two groups with the most similar number of eggs laid were paired for future tests. One of this pair was used for the hexane control and the other for the hexane extract. Three replicates were made for each testing solution.

(2) Ovipositing test

The test weevils were starved and were denied water for twenty-four hours period to the test to reduce the possible effect left from the last feeding. Two six inch long alfalfa plants (with leaves) were dipped in the hexane extract of red clvoer or the hexane control and then air dried for 10 minutes. The cotton strips and the stems were fixed by the same method used in the preliminary study to a small vial and then placed into a cardboard storage box which contained a group of starved alfalfa weevils.

Fresh alfalfa stems were changed every Monday, Wednesday and Friday for a three week test period and the eggs in the stems were counted with the help of a microscope.

(C) Solubility tests of the hexane extract of red clover

By using a rotary evaporator the hexane extract was concentrated from a volumn of 40 ml to 1 ml. The concentrat hexane extract was extracted twice with 5 ml of 5% hydrochloric acid, stirred vigorously and the organic layer separated from the aqueous one. The separated aqueous layer was made basic solution. This solution was then extracted twice with 5 ml of hexane to

extract the insoluble organic compounds. These organic compounds were basic. The hexane extract was then washed until the washings were neutral and then transfered to a weighed Erlemeyer flask and evaporated to dryness and weighed again. The weight difference was the weight of material soluble in acid.

The concentrated hexane extract left after the acid extraction was extracted twice with 5 ml of 5% sodium hydroxide to find out if there was any acidic compounds. The collected aqueous layer was acidified and extracted twice with 5 ml of hexane. The hexane layer was washed with water until the washings were neutral and then transferred to a weighed Erlemeyer flask and evaporated to dryness and weighed again.

The organic layer remaining from the sodium hydroxide extraction was then washed until neutral and transferred to a weighed flask and dried and weighed. The weight difference was the material insoluble in both acid and base.

(D) Thin layer chromatography study of the hexane extract of red clover

Forty milliliters of the hexane extract of red clover were evaporated to 1 ml. A spot was made by applying the concentrated hexane extract to a thin layer chromatographic plate, (7 cm x 2 cm) which was activated at 110^{o}C for one and half hours right before being used, and dried. Several solvents were used as developing agents such as hexane, acetone, alcohol, methanol, ethyl ether nitrobenzene and pyridine.

RESULTS AND DISCUSSION

1. Alfalfa Steam Distillate

(A) The identification of compounds from the steam distillate of alfalfa

Of several column packing materials that were tried to separate the first two peaks (Figure 3), Porapaks Q and R mixed in a 4 to 1 ratio was found to be the best. These first two compounds came out along with a fairly large amount of water.

These compounds emerge from a Porapak column in a sequence based upon their polarities and molecular weights. Based upon the compounds previously identified in this laboratory, ethanol, n-propanol and propionaldehyde it was suspected that methanol would come before ethanol and n-propanol, and acetaldehyde would appear before propionaldehyde. This was found to be true.

In order to confirm the identification of these peaks, 10 ml of the alfalfa steam distillate were separately spiked with 10 μl of acetaldehyde and 10 μl of methanol and then injected into a gas chromatograph. The results indicated that the second peak was acetaldehyde and the first peak was methanol because their retention times match well with the peak retention times. This was confirmed by the GC-MS data (Figure 8).

The third unidentified compound was found to be acetone. The intense peaks at 43 and 58 are very similar to that of acetone.

$$CH_3\text{-}\overset{\overset{O}{/\!/}}{C} \quad (43) \qquad CH_3\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_3 \quad (58)$$

An acetone spike of the alfalfa steam distillate injected into the gas chromatograph verified the third compound was acetone.

The retention times of each of these compounds were double checked by

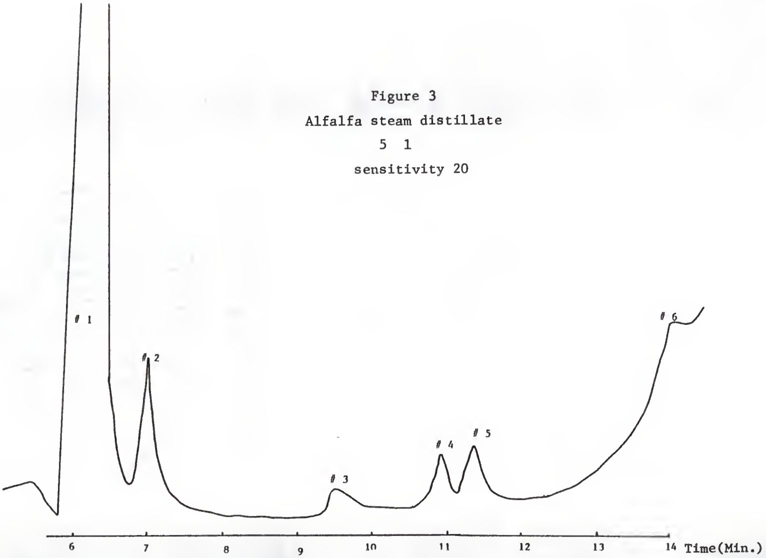


Figure 3
Alfalfa steam distillate
5 1
sensitivity 20

employing a second column packed with Porapak QS. The retention times matched exactly. The gas chromatograms and the mass spectrograms are shown in Figures 3, 4, 5, 6, 7, and 8.

(B) Standard solutions of identified compounds

The relative concentrations of the individual compounds in the alfalfa steam distillate were determined. After several injections of known concentrations of a single compound, the peak areas were calculated and plotted as the concentration. From the peak areas on the chromatogram of the alfalfa steam distillate, the relative individual concentrations of these six identified compounds were obtained. The amount of these compounds present in 100 ml of alfalfa steam distillate were then calculated. In 100 ml of alfalfa steam distillate, they were 3.8 μl for acetaldehyde, 22.2 μl for methanol, 0.8 μl for acetone, 2 μl for n-propanol, 3.0 μl for propionaldehyde and 1.8 μl for ethanol. These amounts of a particular compound were dissolved in 100 ml of distilled water as standard solutions (Table 8). For example, the standard methanol solution was 22.2 μl in 100 ml of distilled water.

Because of the high volatility of acetaldehyde, it was prepared by mixing it with water 1 to 1 first and then making further dilutions as required.

(C) Olfactory orientation test......Feeding test

To test the response of alfalfa weevils, toward various dilutions of the above standard solutions only one identified compound was tested at a time. Each standard solution was prepared in five dilutions and there were six different standards.

The number of the alfalfa weevils attracted toward a test solution and

Figure 4

Alfalfa steam distillate and methanol

3.5 μl

sensitivity 50

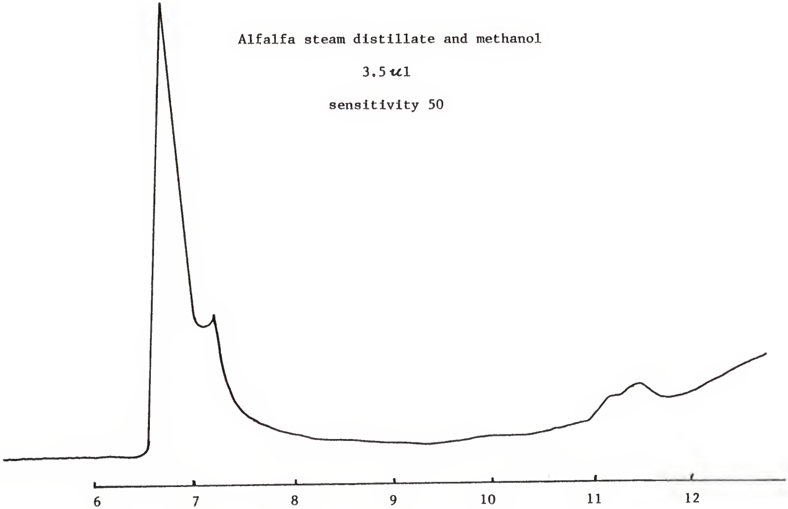


Figure 5

Alfalfa steam distillate, propionaldehyde and acetone

3.5 μl

sensitivity 50

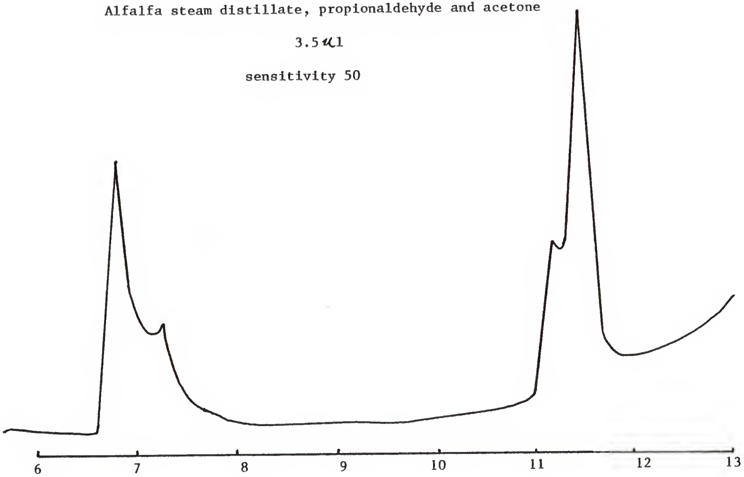


Figure 6

Alfalfa steam distillate and acetone

3.5μl

sensitivity 50

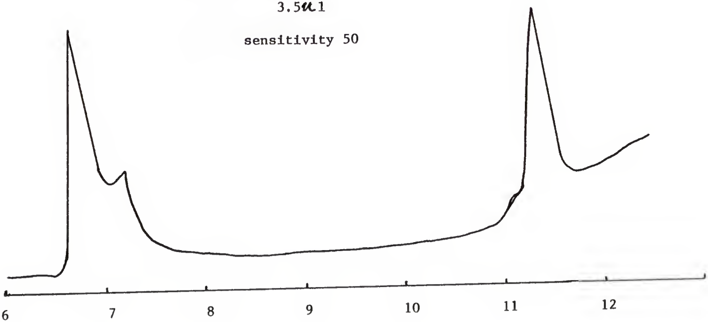


Figure 7

Alfalfa steam distillate and acetaldehyde

3.5 μl

sensitivity 50

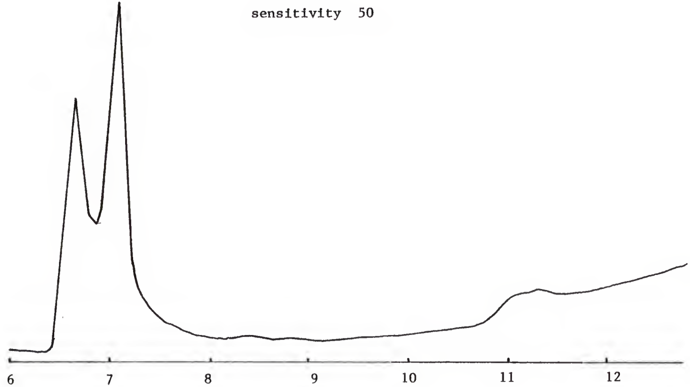
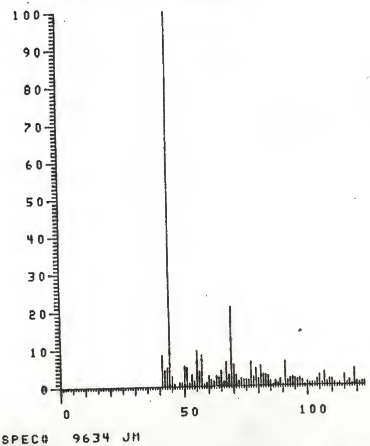


Figure 8

Mass spectra of acetone and acetaldehyde

Mass spectra of Acetaldehyde.



Mass spectra of Acetone.

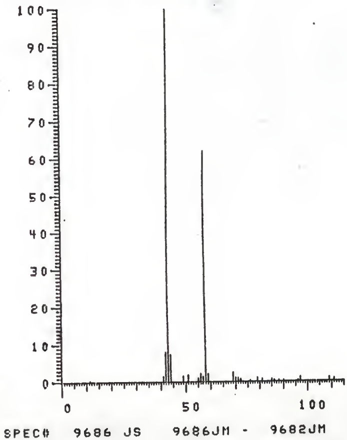


TABLE 8

Standard solution	Amount of pure compound in 100 ml distilled water.		# of molecules per drop.
Acetaldehyde	3.1 µl	2.43 µg	1.7×10^{13}
Acetone	0.8 µl	0.63 µg	3.3×10^{12}
Ethanol	1.8 µl	1.42 µg	7.1×10^{12}
Methanol	22.2 µl	17.73 µg	1.7×10^{14}
n-propanol	2.0 µl	1.87 µg	1.2×10^{13}
Propionaldehyde	3.0 µl	2.42 µg	1.3×10^{13}

water were summed separately for all six replicates and the ratio of these numbers were calculated. The most attractive solution was determined from the ratio of the different concentration levels of each standard solution.

For example, at 0.025% (6.1×10^{-7} ppm) of propionaldehyde, the same concentration level tested at two different dates which were three weeks apart (April 21 and May 12 , 1978) showed quite similar feeding response, 0.7 and 0.74, with a 4% standard diviation.

The ratios for all five dilutions of these six compounds are listed in Table 9 and the highest response of each compound in underlined.

As presented in Table 9, the alfalfa weevil adults were most likely to be attracted by the most dilute solutions. Since in every replicate the weevils provided a choice between the test solution and water (choice test), it was possible to compare these ratios between different test solutions.

An open cage test was used by Byrne[36], in which alfalfa weevils were tested

TABLE 9

Compounds	Ratio of number of weevils attracted to the test solutions as compared to water control .				
	0.0125%	0.025%	0.05%	0.1%	100%
Acetaldehyde	6.17	2.83	1.56	0.91	0.74
Acetone	4.5	0.5	4.33	0.54	0.20
Ethanol	0.5	0.37	0.5	0.33	0.23
Methanol	2.15	3.0	6.7	2.79	0.267
n-propanol	1.22	0.733	1.16	0.8	0.311
Propionaldehyde	1.25	0.74(0.7)	2.2	0.27	0.167

by offering only water as a control, then offering the other test solutions one at a time. However, it was found that during the test period which lasted three to four hours, the weevils changed their behavior during the diurual cycle, such as being more active in the morning. This made it hard to compare the results between treatments. Also, in the open cage test the filter paper is placed in the center of the dish and where to release the test weevils so it would not effect the results is a problem. The choice test had the advantage of testing the water response within each treatment, the alfalfa weevils were always released from the center, and then it is possible to compare the response toward the test solutions.

The net response toward the testsolutions could be compared between treatments. Of these six volatile compounds, it was found that the 0.05% methanol solution elucidated the highest feeding response. Acetaldehyde (0.0125%) and

acetone (0.0125%) showed fairly high responses as compared to the others. Ethanol, n-propanol and propionaldehyde preduced no response by the alfalfa weevils at any of these concentrations.

The net response of the alfalfa weevils was plotted against time for the most attractive concentration. The plots are shown in Figures 9, 10, 11, 12, 13 and 14.

(D) Ovipositing study of identified compounds

A wheat germ diet (Table 7) was used to provide adequate nutrition which is essential to maintain the oviposition response. The grouped weevils were fed on the diet cubes, one of the test solutions, (or water as control) and defoliated fresh alfalfa stems. The number of eggs were recorded.

By using the first setup, those individual solutions which dlucidated the maximum feeding response, a mixture ofthese six compounds, and the alfalfa steam distillate were tested and compared to the control group which was fed on diet cubes only. These data (Table 10) were statistically analyzed by applying the F test. The Ho (hypothesis all treatments are the same) could be rejected with an α value up to 20%. That is, the confidence for saying that these treatments are different is less than 80%. The differences between each treatment was not very significant.

By using the second setup the test solutions were the alfalfa steam distillate, the residue from the steam distillation, and a mixture of the alfalfa steam distillate and the residue. The results (Table 11) were analyzed by applying the F test. These test solutions apparantly did not affect the oviposition level. The eggs laid by the alfalfa weevils in each treatment were close to that of control.

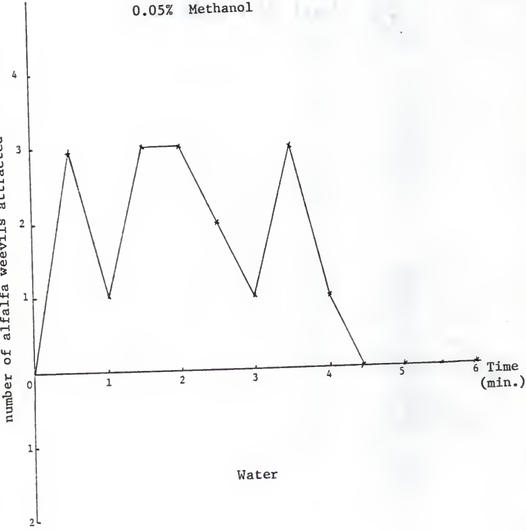

0.05% Methanol
number of alfalfa weevils attracted
4
3
2
1
0
1
2
1
2
3
4
5
6
Time (min.)
Water

Figure 9

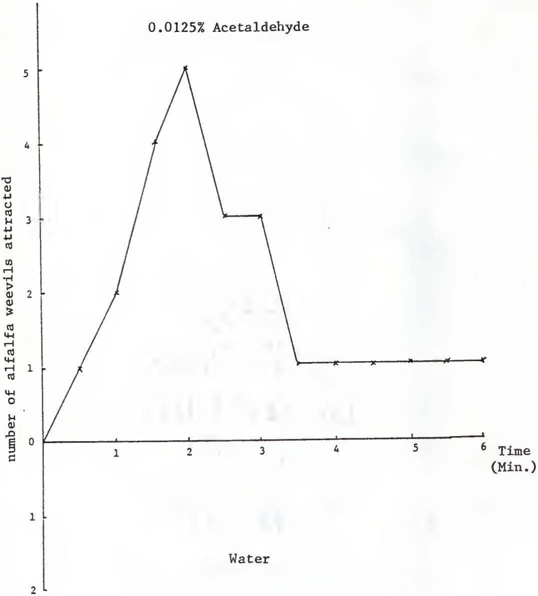

0.0125% Acetaldehyde
number of alfalfa weevils attracted
5
4
3
2
1
0
1
2
1
2
3
4
5
6
Time
(Min.)
Water

Figure 10

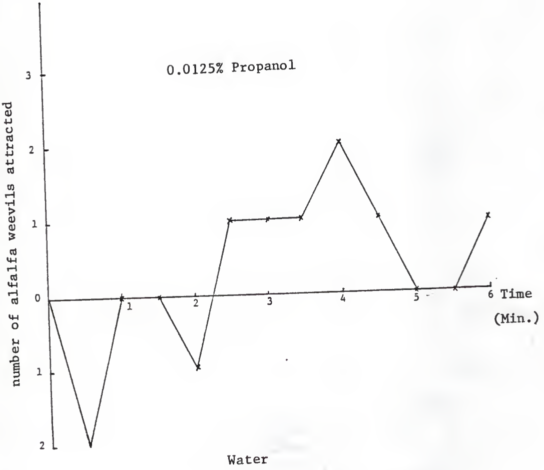

0.0125% Propanol
number of alfalfa weevils attracted
3
2
1
0
1
2
1
2
3
4
5
6 Time
(Min.)
Water

Figure 11

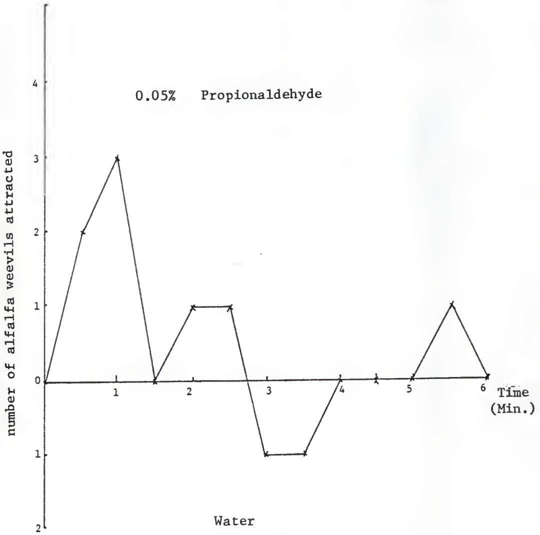

0.05% Propionaldehyde
number of alfalfa weevils attracted
4
3
2
1
0
1
2
1
2
3
4
5
6
Time
(Min.)
Water

Figure 12

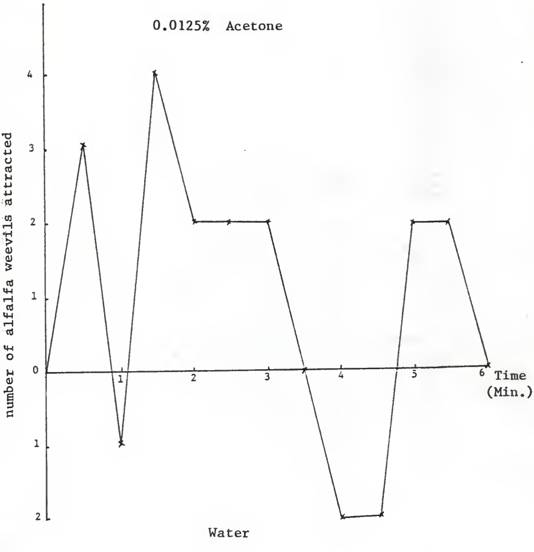

0.0125% Acetone
number of alfalfa weevils attracted
4
3
2
1
0
1
2
1
2
3
4
5
6
Time
(Min.)
Water

Figure 13

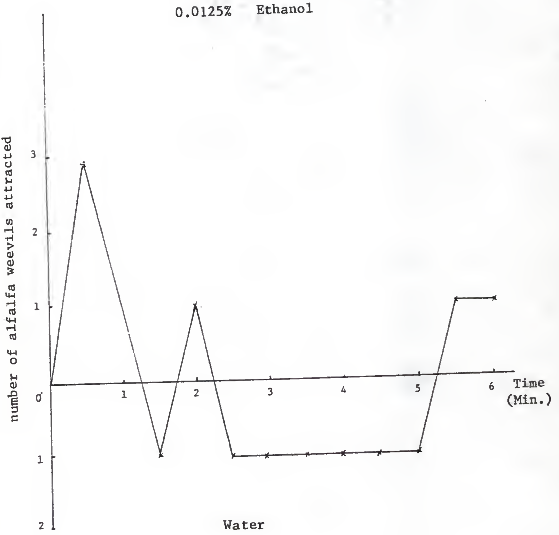

0.0125% Ethanol
number of alfalfa weevils attracted
3
2
1
0
1
2
1
2
3
4
5
6
Time
(Min.)
Water

Figure 14

TABLE 10

Chemicals	Concentrations %	μg/ml	Total eggs laid
Acetaldehyde	0.0125	3.0	77
Acetone	0.0125	0.79	67
Ethanol	0.0125	1.78	79
Methanol	0.05	88.5	166
n-propanol	0.0125	2.34	78
Propionaldehyde	0.0125	12.1	103
Alfalfa	---		1158
Mixture of solvents	---		174
Diet only	---		80

TABLE 11

Treatment	Number of eggs laid
Alfalfa steam distillate	78
Residue from the distillation	108
Alfalfa steam distillate and residue from the steam distillation.	183
Diet	117
Alfalfa	1931

The differences between treatments were not significant. None of these test solutions, including the alfalfa steam distillate and the residue from the steam distillation appeared to contain the bioactive material.

An alfalfa, for which three replicates were conducted at the same time, induced a number of eggs laid that far exceeded that of any other treatment(Table 11). There are at least two explanations why the alfalfa weevils didn't lay as many eggs on the various treatments as they did on alfalfa. First, supposing that a bioactive material had existed, it may have been destroyed during the steam distillation. It is possible that the oviposition stimulant was heat sensitive. Otherwise, the material would have been in either the alfalfa steam distillate or in the residue. If any bioactive material was present and not destroyed, one of these two treatments should have demonstrated a significantly higher level of egg laying. Secondly, it may not be chemicals that stimulate oviposition. Oviposition could be stimulated either by mechanical, tactile or visual stimuli.

2. Hexane Extract of Red Clover

(A) Oviposition Test

The purpose of this research, was to examine the prepared hexane extract of red clover and examine its' effectiveness in the oviposition process.

The data shown in Table 12, 13, and 14 were analyzed statistically by applying the F test. The hexane extract of red clover showed a significant reduction on the egg laying level as compared to the solvent alone. That suggested strongly that an oviposition inhibitor for the alfalfa weevil is present in red clover and that this bioactive material could be extracted by hexane.

TABLE 12

Treatment	Number of eggs laid	$F_{Calculated}$	$F_{(1, 30, 0.05)}$
Hexane extract of red clover	82	5.35	4.7
Hexane control	259		

TABLE 13

Treatment	Number of eggs laid	$F_{Calculated}$	$F_{(1, 38, 0.05)}$
Hexane extract of red clover	72	7.1	4.1
Hexane control	406		

An example calculation for the F test analysis is shown on next page.

TABLE 14

Treatment	Number of eggs laid	$F_{Calculated}$	$F_{(1, 70, 0.05)}$
Hexane extract of red clover	649	8.16	3.98
Hexane control	1232		

Example on the calculation of the F test:

Data: number of eggs of two treatments

Hexane control: 121, 64, 43, 17, 24,28, 24, 20, 30, 8, 27, 8, 94, 26,64, 29, 81, 21, 37, 11, 0, 15, 60, 28, 38, 70, 28, 0, 27, 22, 27, 14, 0, 44, 23, 69.

Hexane extract: 33, 57, 8, 43, 8, 23, 6, 8, 0, 6, 4, 29, 47,21, 10, 11, 21, 14, 0, 7, 0, 9, 30, 16, 82, 70, 15, 6, 18, 4,9, 3, 0, 0, 28. 4.

AVO TABLE[42]

Source degree of freedom	Sum of square	Mean of square	F_{value}
Between 2 - 1 = 1	4720	4720	8.16
Within 2 x (36 - 1) = 70	40488	578.4	
Total 70 + 1 = 71	45208		

Sum of square (total) = $X_{ij}^{2} - (X..)^{2}/N$

$$X_{ij}^2 = 33^2 + 47^2 + \ldots\ldots. + 121^2 + 94^2 +\ldots..$$

$$= 94349$$

$$(X..)^2/N = (33 + \ldots+ 121 + 94 +\ldots)/36 \times 2$$

$$= 49141$$

$$\text{S.S. total} = 94349 - 49141 = 45208$$

$$\text{Sum of square (between)} = \text{uncorrected between} - (X..)^2/N$$

$$\text{Uncorrected between} = (X_i)^2/36$$

$$= 53861$$

$$\text{S. S. between} = 53861 - 49141$$

$$= 4720$$

$$\text{S. S. within} = 45208 -4720$$

$$= 40488$$

Mean of square = sum of square / degree of freedom

Mean of square (between) = 4720

Mean of square (within) =578.4

$$F_{value} = \text{mean of square (between) / mean of square (within)}$$

$$= 4720/ 578.4$$

$$= 8.16$$

$F_{(1,\ 70,\ 0.05)} = 3.98$[42]

Because the F value from the calculation is larger than taht from the Table, Ho (hypothesis that two treatments are equal) can be rejected within 5%, that is, we have 95% confidence to say that these two treatments, hexane control and hexane extract of red clover, are different.

In this experiment, it was also found that if the alfalfa plants were dipped into the hexane solutions (hexane control or hexane extract) too long, the plants would dry out easily, and this would stop the weevils from feeding a and decrease theegg laying level. The optimum dipping time is about 5 seconds. The results shown in Table 14 and the F test calculation were the ones with a preliminary study of the alfalfa weevils' history.

From these results, the oviposition tests on the hexane extract of red clover provided strong evidence for the presence of an ovipositing inhibitor in the water and ether washed hexane extract of red clover. This extract was further analyzed by solubility tests, thin layer chromatography and high performance liquid chromatography.

(B) Solubility tests of the hexane extract of red clover

The results (the weight difference between theweighed flask and the weight of the flask after dryness) indicated that there were neither acid soluble material nor basic soluble substances present in the hexane extract of red clover. It was shown that the compound or compounds extracted by hexane was a neutral compound.

(C) Thin layer chromatography study

When the plates were developed in hexane one spot was found at the origin, a second long tailed spot was found with an R_f value of 0.55. When developed with ethyl acetate, nitrobenzene, ethanol and pyridine, only one long tailed spot was found with R_f values at 0.7, 0.45, 0.28 and 0.4 respectively. The charring agent was 50% sulfuric acid.

Attempts to separate the extract by gas chromatography techniques were

unsuccessful. Only a solvent peak could be seen. Different column packing materials were tried and none of these columns seemed to work. The compound present in the hexane extract of the red clover probably was a high molecular weight compound. High pressure liquid chromatography was tried and three peaks were separated from the solvent peak. A C_{18} column packing material and a 70/30 methanol/water (v/v) solvent system were used. The retention times were very close, 19 minutes, 19½ minutes and 20 minutes.

CONCLUSION

From the results listed in Table 8, the conclusion can be made that acetaldehyde, acetone and methanol are three feeding stimulants present in the fresh alfalfa steam distillate. However, none of the six identified compounds were ovipositing stimulants. Also, it could be concluded that the ovipositing stimulant was not present in either the fresh alfalfa steam distillate or the residue left after the steam distillation. Byrne[36] reported that there were no ovipositing stimulants in the fresh alfalfa steam distillate. At this point, two results did coincident to each other.

FUTURE WORK

It might be possible that chemical ovipositing stimulants are present in the alfalfa, but they are not in the systems studied. It may be easier to find a way to control the alfalfa weevils by studying the deterrent in the red clover. There is strong evidence to support the presence of the deterrent in the red clover.

The hexane extract of red clover could be applied to a thin layer plate and the separated compounds examined. A better TLC separation is required such as a multiple development process. These purified components could then be fed to the ovipositing alfalfa weevils. From the ovipositing test, the bioactive portion of the hexane extract might be determined. Also, the thin layer plate after development can be examined by different specific spray agents, such as ninhydrin for amino groups and rhodamine 6 G for lipids. Then by either thin layer chromatography or column chromatography the components can be purified.

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THREE FEEDING STIMULANTS

IN THE ALFALFA STEAM DISTILLATE FOR

THE ALFALFA WEEVILS, (HYPERA POSTICA (GYL.))

by

RITA HWAY-YING CHIOU

B. S., National Taiwan Normal University
Taipei, Taiwan, 1972

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1979

ABSTRACT

The alfalfa weevil, now found in all parts of this country infesting alfalfa in nearly every state, is one of the most important pests of alfalfa.

The identification of (1) the attractant in alfalfa, and (2) the deterrent in red clover could be used to develope alfalfa cultivates resistant to the attack of the alfalfa weevils.

The compounds present in the steam distillate of alfalfa were identified by the GC-MS spectroscopic data and matched with the retention times of known compounds by using two different column packing material.

Choice test was used to bioassay the identified alfalfa components and found out that methanol, acetone and acetaldehyde are three feeding stimulants for the alfalfa weevils.

A preliminary study of red clover hexane extract showed strong evidence of the presence of an oviposition deterrent.